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**March 21, 2003**

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**APPLICATION NUMBER:** ***60/371,442***
**FILING DATE:** ***April 10, 2002***

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Appr.



**Practitioner's Docket No. 0512** ***PATENT***

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Tracy Marie HANDEL, Amanda PROUDFOOT, Marie KOSCO-VILBOIS

For: NOVEL ANTAGONISTS OF MCP PROTEINS

**Box Provisional Patent Application**
**Assistant Commissioner for Patents**
**Washington, D.C. 20231**

COVER SHEET FOR FILING PROVISIONAL APPLICATION
(37 C.F.R. § 1.51(c)(1))

*WARNING.* *"A provisional application must also include the cover sheet required by 37 C.F.R. 1.51(c)(1) or a cover letter identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under paragraph (b) [nonprovisional application] of this section. " 37 CFR. 1.53(c)(1). See also M.P.E.P. § 201.04(b), 6th ed,. Rev. 3.*

*NOTE:* *"A complete provisional application does not require claims since no examination on the merits will be given to a provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional application. " Notice of December 5,1994, 59 Fed. Reg. 63,951, at 63,953.*

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CERTIFICATION UNDER 37 C.F.R. § 1.10
*(Express Mail label **number is mandatory**.)*
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I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on April 10, 2002, in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. § 1.10 Mailing Label Number ET852529563US addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Gregory Butler
*(type or print name of person mailing paper)*

Gregory B. Butler
**Signature of person mailing paper**

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*"Since the filing of correspondence under § 1. 10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will not be granted on petition. "Notice of Oct.14, 1996, 60 Fed. Reg..56,439, at 56,442.*

*"Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure. "* Notice of April *14,* 1995, 60 *Fed.* Reg. 20,195. *at* 20,209.

*NOTE: "A provisional application is not entitled to the right of priority under* 35 US.C 119 *or* 365(a) *or § 1.55, or to the benefit Of an earlier filing date under 35* U.S.C 120,121 *or 365(c) or § 1.* 78 of any *other application.* No *claim of priority under § 1. 78(a)(3)* may be *made* in *a design application based on a provisional application.* No *request under § 1. 293 for a statutory invention registration may be filed in a provisional application. The requirements* of §§ 1.821 *through 1.825 regarding application disclosures containing nucleotide andlor amino acid sequences are not mandatory for provisional applications. "* 37 CFR. § *1.53(c)(3).*

*NOTE: "No information disclosure statement may be filed in a provisional application. " 37 CFR. § 1.51(d). "Any info disclosure statement filed in a provisional application would either be returned or disposed of at the convenience of the Office" Notice of December 5. 1994, 59 Fed Reg. 63,591, at 63,594.*

*NOTE: "No amendment other than to make the provisional application comply with the patent statute and all applicable regulations, may* be *made to the provisional application after the filing date* of *the provisional application. " 37 CF.P, 1..* 53 *(c).*

*WARNING: A provisional application maybe abandoned by operation af 35US.C 111(b)(5)on a Saturday, Sunday or Federal holiday within the District of Columbia, in which case, a nonprovisional application claiming benefit of the provisional application under 35 U.S. C 119(e) must be filed no later than the preceding day that is not a Saturday, Sunday, or Federal holiday within the District of Columbia. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,202.*

**This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R § *1.*51 (c)(1)(i).**

1. **The following comprises the information required by 37 C.F.R. § 1.5 l(c)(1):**

2. **The name(s) of the inventor(s) is/are (37 C.F.R. § *1.5* 1 (c)(1)(ii)):**

*NOTE: "If the correct inventor or inventors are not named on filing, a provisional application without a cover sheet under § 1.51(c)(1), the later submission of a cover sheet under § 1.51(c)(1) during the pendency of the application will act to correct the earlier identification of inventorship. " 37 CFR. § 1.4809(2).*

*NOTE: "The naming of itiventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al 'will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee se forth in § 1. I 7(i) is filed which sets forth the reasons the delay in supplying the names should be excused Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S. C I I l(a) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C 111(a)[,] application must have at least one inventor in common with the provisional application. " Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.*

*The term "invention " is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention " disclosed in the provisional application. @the "invention " has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.45(c) states that "if multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application. " All that § 1.45(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. "When applicant has determined what the invention is by the filing of the 35 U.S.C I I l(a) application, that is the time when the correct inventors must be named. The 35 U.S.C I 11a) application must have an inventor in common with the provisional application in order for the 35 U.S. C I I l(a) application to be entitled to claim the benefit of the provisional application under 35 U.SC 119(e). Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,108. See 37 CFR. § 1.53.*

| | | | |
|---|---|---|---|
| NAME | | | |
| 1. | Tracy | Marie | HANDEL |
| | GIVEN NAME | MIDDLE INITIAL OR NAME | FAMILY (OR LAST) |
| NAME | | | |
| 2. | Amanda | | PROUDFOOT |
| | GIVEN NAME | MIDDLE INITIAL OR NAME | FAMILY (OR LAST) |
| NAME | | | |
| 3. | Marie | | KOSCO-VILBOI |
| | GIVEN NAME | MIDDLE INITIAL OR NAME | FAMILY (OR LAST) |

3. Residence address(es) of the inventor(s), as numbered above (3 7 C.F.R. § *1.5* 1 (c)(1)(iii)):

   1. 225 Clifton Street, Apt 312, Oakland, California, United States of America
   2. Sous Chens, 74140 Chens-sur-Leman, France
   3. Le Cret, 74270 Minzier, France

4. The title of the invention is (3 7 C.F.R. § *1.5* 1 (c)(1)(iv)):

   NOVEL ANTAGONISTS OF MCP PROTEINS

5. The name, registration, customer and telephone numbers of the practitioner (if applicable) are (37 C.F.R. § 1.5 1 (c)(1)(v)):

   Name of practitioner: Gregory B. Butler

   Reg. No. 34,558 Tel. (781) 681-2796

   Customer No.

   *(complete the following, if applicable)*

   [ ] A power of attorney accompanies this cover sheet.



6. The docket number used to identify this application is (3 7 C.F.R. § *1.5* 1 (c)(1)(vi)):

   Docket No. 0512

(Cover Sheet for Filing Provisional Application-page 3 of 5)

7. The correspondence address for this application is (3 7 C.F.R. § 1.5 1 (c)(1)(vii)):
Gregory B. Butler, PhD, Esq.
SERONO REPRODUCTIVE BIOLOGY INSTITUTE,
One Technology Place, Rockland, MA 02370

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with agency of the U.S. Government. (37 C.F.R. § *1.5* 1 (c)(1)(viii)).

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

[X] No
[ ] Yes

The name of the U.S. Government agency and the Government contract number are:

9. Identification of documents accompanying this cover sheet:

A. Documents required by 3 7 C.F.R. § § 1.51 (c)(2)-(3):

| | | |
|---|---|---|
| Specification: | No. of pages: 32 | (including cover sheet) |
| Drawings: 8 | No. of sheets: 8 | |

B. Additional documents:

[X] Claims: No. of claims: 17 , 2 pages

*Note: See 37* CFR. § *1.51.*

[ ] Power of attorney
[ ] Small entity statement
[ ] Assignment
[ X] Other - I page abstract

*NOTE: Provisional applications may be filed in a language other than English as set forth in existing § 1. 52(d). However, an Engli language translation is necessary for security screening purposes. Therefore, the PTO will require the English langua translation and payment of the fee mandated in §1.52(d)in the provisional application .Failure to timely submit the translation response to a PTO requirement will result in the abandonment of the provisional application. If a 35 U.S.C. 1111(a) applicati is filed without providing the English language translation in the provisional application, the English language translation w be required to be supplied in every 35 U.SC 1 11(a) application claiming priority of the non-English language provision application. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.*

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1. 16(k), is $160.00, for other than a small entity, and $80.00, for a small entity.

[ ] Applicant is a small entity.
[X] Applicant is not a small entity.

*NOTE: "A .....statement in compliance with existing § 1.27 is required to be filed in each provisional applicatic in which it is desired to pay reduced fees. " Notice of .4prit 14, 1995, 60 Fed. Reg. 20,195. at -710,197.*

1 1. Small entity statement

[ ] The statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27 is(are) attached.

12. Fee payment

[X] Fee payment in the amount of $160.00 is being made at this time.

[ ] No filing fee is to be paid at this time. (This and the surcharge required by 3 7 C.F.R. § 1. 16(l) can be paid subsequently.)

13. Method of fee payment

[ ] Check in the amount of $ 160.00
[ X ] Charge Account No. 501365 in the amount of $160.00
A duplicate of this Cover Sheet is attached.
Please charge Account No. 501365 for any fee deficiency.

Gregory B. Butler

**Signature of Practitioner**

Date: April 10, 2002

Reg. No.: 34,558

Gregory B. Butler
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Customer No.:

# NOVEL ANTAGONISTS OF MCP PROTEINS

## FIELD OF THE INVENTION

This patent describes novel antagonists of MCP proteins, and in particular of human MCP-1, generated by mutagenising MCP proteins.

## BACKGROUND OF THE INVENTION

Chemokines are small secreted pro-inflammatory proteins, which mediate directional migration of leukocytes from the blood to the site of injury. Depending on the position of the conserved cysteines characterizing this family of proteins, the chemokine family can be divided structurally in C, C-C, C-X-C and C-$X_3$-C chemokines, to which corresponds a series of membrane receptors (Baggiolini M et al., 1997; Fernandez EJ and Lolis E, 2002). Usually chemokines are produced at the site of injury and cause leukocyte migration and activation, playing a fundamental role in inflammatory, immune, homeostatic and angiogenic processes. These molecules, therefore, offer the possibility for therapeutic intervention in diseases associated to such processes, in particular by inhibiting specific chemokines and their receptors at the scope to preventing the excessive recruitment and activation of leukocytes (Baggiolini M, 2001; Loetscher P and Clark-Lewis I, 2001; Godessart N and Kunkel SL, 2001).

Monocyte chemoattractant protein 1 (from now on, MCP-1) is a member of the CC chemokine family also known under various names such as Small Inducible Cytokine A2 (SCYA2), Monocyte Chemotactic And Activating Factor (MCAF), Monocyte Secretory Protein Je, Monocyte Chemotactic Factor, and HC11. This chemokine is capable of promoting the recruitment of monocytes and basophiles in response to injury and infection signals in various inflammatory diseases, different types of tumors, cardiac allograft, AIDS, and tuberculosis (Gu L et al., 1999). The physiological activities associated to MCP-1 have been extensively studied by means of transgenic animals, which allowed to demonstrate that MCP-1 controls not only monocyte recruitment in inflammatory models, but also the expression of cytokines related to T helper responses and the initiation of atherosclerosis (Gu L et al., 2000; Gosling J et al, 1999; Lu B et al, 1998).

Structurally, MCP-1 consists of a N-terminal loop and a β sheet overlaid by an α-helix at the C-terminal end (Handel TM et al., 1996; Lubkowski J, et al., 1997).

Structurally and functionally homologous proteins have been identified and called MCP-2, MCP-3, and MCP-4 (Proost P et al., 1996; Blaszczyk J et al., 2000). The literature provides many examples of structure-activity studies (Gong JH and Clark-Lewis I, 1995; Zhang et al., 1996; Steitz SA et al., 1998; Gu L et al., 1999; Hemmerich S et al., 1999; Seet BT et al., 2001) in which MCP-1 mutants have reduced activity and/or affinity for the receptor or other binding proteinshave been obtained by expressing N-terminal truncations (as in many other chemokines), or single mutations at residues 3, 8, 10, 13, 15, 18, 19, 24, 28, 30, 37, 38, and 39 (following the numbering of mature human MCP-1).

Chemokines interact with Proteoglycans (PGs) and glycosaminoglycans (GAGs) a feature common to many cell-signaling soluble molecules (interleukins, growth factors). Proteoglycans are negatively charged proteins that are posttranslationally modified by the addition of glycosaminoglycan side chains at serine residues. Clusters of basic residues (mainly Arginine and Lysine) allow proteins to interact with GAGs, which commonly are characterized by the disaccharide repeats such as heparin, chondroitin sulfate, heparan sulfate, dermatan sulfate, and hyaluronic acid). PGs and GAGs can be present on membrane surfaces as well as soluble molecules, probably at the scope to protect this molecule from proteolysis in the extracellular environment. It has been also proposed that GAGs may help the correct presentation of cell signaling molecules to their specific receptor and, eventually, also the modulation of target cell activation. In the case of chemokines, the concentration into immobilized gradients at the site of inflammation and, consequently, the interaction with cell receptors and their activation state seem to be modulated by the specific GAGs. The interaction with GAGs and the formation of these gradients have been clearly demonstrated for many chemokines, including MCP-1, measuring the relative affinity. Therefore, it has been suggested that the modulation of the such interactions may represent a therapeutic approach in inflammatory disease (Hoogewerf AJ et al., 1997; Kuschert G et al., 1999; Ali S et al., 2001; Patel D et al., 2001).

However, the structural requirements and functional effects of GAGs / MCP-1 interactions have been poorly studied. It is known that GAGs can modulate the activity and production of MCP-1 secreted from endothelial cells (Douglas MS et al., 1997). It has been also reported that substitution of Lysine 58 and Histidine 66 with Alanines in the C-terminal of MCP-1, prevents GAG binding without affecting receptor binding, $Ca^{2+}$ influx, or chemotactic activity (Chakravarty L et al, 1998), but there is no disclosure in

the prior art of which may be other GAG binding sites of MCP-1, and which *in vivo* effects can be consequent to their elimination. Even though extensive studies have been performed on some chemokines, it is not possible to anticipate, on the basis of the sequence homology, which residues have to be modified with non-conservative substitutions to impair GAG binding, and which effects can be obtained, since there is a significant structural diversity of GAG binding domains in chemokines (Chakravarty L et al, 1998; Lortat-Jacob H et al., 2002).

## SUMMARY OF THE INVENTION

It has been found that a dibasic site at the N-terminal of human MCP-1 (Arginine 18, Lysine 19) is responsible for the interaction of MCP-1 with GAGs. The elimination of this site by non-conservative substitutions (for example, with Alanine) allows to generate MCP-1 mutants having not only have a reduced tendency to interact with GAGs, but a surprising *in vivo,* dose-related antagonistic activities on MCP-1. Such evidences can be exploited to use mutants of MCP-1, and of other MCP proteins, as antagonists of the corresponding MCP protein. Compounds prepared in accordance with the present invention can be used to inhibit the migration and activation of leukocytes expressing their receptors, thereby providing useful therapeutic compositions for use in the treatment of diseases related to excessive or uncontrolled leukocyte migration, such as inflammation and autoimmune diseases. Other features and advantages of the invention will be apparent from the following detailed description.

## DESCRIPTION OF THE FIGURES

Figure 1: (A) amino acid sequences of human and mutated MCP-1 proteins as expressed and tested in the Examples (mutated amino acids are underlined). The N-terminal methionine in MCP-1WT* and MCP-1WT*2A was removed during purification by aminopeptidase treatment to avoid any interference on the activity of the protein due to this additional residue. (B) Alignment of the mature forms of human MCP-1 (SWISSPROT Acc. N° P13500), MCP-2 (SWISSPROT Acc. N° P80075), MCP-3 (SWISSPROT Acc. N° P80098), and MCP-4 (SWISSPROT Acc. N° Q99616). Boxed Residues are the one identified in the Examples (residues 18 and 19) and in the prior art (residues

58 and 66) as being involved in the binding with GAGs. Underlined residues are other basic residues conserved amongst human MCP proteins.

Figure 2: graph representing the results of the heparin binding assay performed with [$^3$H]-heparin and, as chemokine, either MCP-1WT* (O) or MCP-1WT*2A (●).

Figure 3: graph representing the results of the equilibrium competition receptor binding assay performed by monitoring the displacement of [$^{125}$I]-MCP-1 from CCR2-expressing CHO membranes consequent to the addition, as chemokine, of hMCP-1 (□), MCP-1WT* (O), or MCP-1WT*2A (●).

Figure 4: graph representing the results of the transwell chemotaxis assay performed using THP-1 cells and, as chemokine, hMCP-1(□); MCP-1WT*, (O) or MCP-1WT*2A (●).

Figure 5: graph summarizing the results of the peritoneal cells recruitment assay, performed in mice using MCP-1WT* and/or MCP-1WT*2A. The concentrations of MCP-1WT*2A showing a statistically significant inhibition activity on the number of cells recruited by MCP-1WT* are indicated with *.

Figure 6: graph summarizing the results of the delayed contact hypersensitivity assay. Mice were treated with 0.5 mg/kg MCP-1WT*2A (■) or with vehicle only (□). The effect is measured in terms of ear swelling volume in the following days.

Figure 7: graph summarizing the effects on body weight of mice untreated (□) or treated with bleomycin to induce lung fibrosis. The treated mice received as well an intraperitoneal administration of 0.25 mg/kg MCP-1WT*2A (●) or vehicle only (O). The indicated weight is an average value.

Figure 8: graph comparing the fibrosis levels in untreated and bleomycin-treated mice, which were additionally treated with vehicle only, or with an intraperitoneal administration of 0.25 mg/kg MCP-1WT*2A. Fibrosis levels were measured either spectroscopically (top) or histologically (bottom) as described in the Examples.

## DETAILED DESCRIPTION OF THE INVENTION

In view of the above mentioned evidences in the prior art, there is no indication that a specific dibasic site in the N-terminus of human MCP-1 defines a GAG binding site, and that the non-conservative substitution of the residues in this site leads to molecules having antagonistic activity on MCP-1. Moreover, given the conservativity of this dibasic site amongst known MCP proteins, as well as of other basic residues

known to be involved in GAG binding, it can be inferred that the antagonists of MCP proteins can be obtained by non-conservative substitutions in the residues corresponding to the ones functionally characterized in human MCP-1.

The main object of the present invention is to provide novel antagonists of MCP proteins consisting of mutants of MCP proteins in which the following combinations of basic residues, numbered on the sequence of human mature MCP-1, are substituted to Alanine, Glycine, Serine, Threonine, Proline, Aspartic acid, or Asparagine:

a) 18 and 19;

b) 18 and/or 19, together with 58;

c) 18 and/or 19, together with 66;

d) 18 and/or 19, together with 58 and 66;

e) 18 and/or 19, together with one or more of the following: 24, 44, 49, 75.

The present patent application provides surprising *in vivo* and *in vitro* data obtained with a novel recombinant MCP-1 mutant in which Arginine 18 and Lysine 19 were substituted with Alanines, which is a particular example of the combinations described above. These evidences, combined with the knowledge on the sequence and the structure of other highly conserved MCP proteins suggests that this dibasic site can play not only a general role in MCP proteins biological activity, but also can be modified accordingly in these homologous proteins to obtain antagonist molecules.

The basic residues which have to be mutated in a non-conservative manner in MCP proteins to obtain molecules having antagonistic properties are essentially both residues in positions 18 and 19, at least one of the basic residues in position 18 and 19 combined with at least one of the basic residues already known to be involved in GAG binding, such as 58 and 66 (Chakravarty L et al., 1999), or at least one of the basic residues in position 18 and 19 combined with at least one of the other basic residues which are conserved in all human MCP proteins (fig. 1B). The amino acid replacing the basic residue is preferably a non-polar, small amino acid like Alanine or Glycine, but other amino acids are appropriate, provided that they have a charge and dimension which poorly interfere with the structure of the protein and, at the same time, are incompatible with GAG binding, for example Serine, Threonine, Proline, Aspartic acid, or Asparagine.

Therefore the main object of the present invention is to provide mutants of MCP proteins which contain a combination of the mutations defined above, and which act as antagonists of MCP proteins.

The term "antagonist of MCP proteins" means any molecule, which acts as antagonist to the corresponding mature full-length, naturally-occurring (wild-type) MCP protein. MCP-1 antagonists known in the art involves modifications

In the sense of the present application, the term MCP proteins include human MCP-1, human MCP-2, human MCP-3, and human MCP-4 (fig. 1B; the legend indicates the corresponding SWISSPROT accession numbers), as well as any other protein having at least 70%, preferably 80%, and more preferably 90% of homology with human mature MCP-1, MCP-2, MCP-3, or MCP-4, and contain a basic, positively charged amino acid (Arginine, Lysine, or Histidine) in all the positions identified above. Acceptable substitutions should involves other residues not involved in GAG binding, like the substitution of Methionine 64 with an Isoleucine shown in the examples (MCP-1WT*2A; SEQ ID NO: 3) to improve purification without altering essential properties of human MCP-1.Another object of the present invention is therefore an MCP-1 antagonists having the sequence corresponding to MCP-1WT*2A (SEQ ID NO: 3).

Further objects of the present invention are antagonists of MCP proteins selected from:

a) active mutants of the above defined mutants of MCP proteins in which one or more amino acid residues have been added, deleted, or substituted without interfering with the antagonistic activity;

b) peptide mimetics designed on the sequence and/or the structure of polypeptides or peptides of (a);

c) polypeptides or peptides comprising the amino acid sequence of (a) or (b), and an amino acid sequence belonging to a protein sequence other than the corresponding MCP protein;

d) active fractions, precursors, salts, or derivatives of (a), (b), or (c).

The antagonistic properties of MCP mutants defined above, and exemplified in the present patent application using MCP-1WT*2A as MCP-1 antagonist, can be maintained, or even potentiated, in the active mutants. This category of molecules includes natural or artificial analogs of said sequence, wherein one or more amino acid residues have been added, deleted, or substituted, provided they display the same biological activity characterized in the present invention at comparable or higher levels, as determined by means known in the art and dislcosed in the Examples below. Natural analogs are intended the corresponding sequences of MCP proteins identified in humans or in other organisms, like mouse MCP-1 (SWISSPROT Acc. N° P10148).

Artificial analogs are intended peptides prepared by known chemical synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable thereof, which provide a finite set of substantially corresponding mutated or shortened peptides or polypeptides which can be routinely obtained and tested by one of ordinary skill in the art using the teachings presented in the prior art and in the Examples of the present patent application.

In accordance with the present invention, preferred changes in these active mutants are commonly known as "conservative" or "safe" substitutions, and involve non-basic residues. Conservative amino acid substitutions are those with amino acids having sufficiently similar chemical properties, in order to preserve the structure and the biological function of the molecule. It is clear that insertions and deletions of amino acids may also be made in the above defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under ten, and preferably under three, and do not remove or displace amino acids which are critical to the functional conformation of a protein or a peptide.

The literature provide many models on which the selection of conservative amino acids substitutions can be performed on the basis of statistical and physico-chemical studies on the sequence and/or the structure of natural protein (Rogov SI and Nekrasov AN, 2001). Protein design experiments have shown that the use of specific subsets of amino acids can produce foldable and active proteins, helping in the classification of amino acid "synonymous" substitutions which can be more easily accommodated in protein structure, and which can be used to detect functional and structural homologs and paralogs (Murphy LR et al., 2000). The synonymous amino acid groups and more preferred synonymous groups are those defined in Table I.

Active mutants produced by substitutions made on the basis of these teachings, as well as active mutants wherein one or more amino acids were eliminated or added, are amongst the objects of the present invention, that is, novel mutants of MCP proteins having poor GAG binding properties and antagonistic activity on the corresponding MCP protein, comparable to the ones of the initially selected mutants, or even improved if possible.

The above described alternative compounds are intended to comprehend molecules with changes to the sequence of the mutants of MCP proteins defined above which do not affect the basic characteristics disclosed in the present patent application, particularly insofar as its ability as antagonists is concerned. Similar compounds may

result from conventional mutagenesis technique of the encoding DNA, from combinatorial technologies at the level of encoding DNA sequence (such as DNA shuffling, phage display/selection), or from computer-aided design studies, followed by the validation for the desired activities as described in the prior art and in the Examples below.

Specific antagonists can be obtained in the form of peptide mimetics (also called peptidomimetics) of the above defined MCP mutants, in which the nature of peptide or polypeptide has been chemically modified at the level of amino acid side chains, of amino acid chirality, and/or of the peptide backbone. These alterations are intended to provide MCP antagonists having similar or improved therapeutic, diagnostic and/or pharmacokinetic properties.

For example, when the peptide is susceptible to cleavage by peptidases following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a non-cleavable peptide mimetic can provide a peptide more stable and thus more useful as a therapeutic. Similarly, the replacement of an L-amino acid residue is a standard way of rendering the peptide less sensitive to proteolysis, and finally more similar to organic compounds other than peptides. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl, succinyl, methoxysuccinyl, suberyl, adipyl, azelayl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazelayl, methoxyadipyl, methoxysuberyl, and 2,4,-dinitrophenyl. Many other modifications providing increased potency, prolonged activity, easiness of purification, and/or increased half-life are known in the art (WO 02/10195; Villain M et al., 2001). Preferred alternative, "synonymous" groups for amino acids included in peptide mimetics are those defined in Table II.

The techniques for the synthesis and the development of peptide mimetics, as well as non-peptide mimetics, are well known in the art (Sawyer TK, 1997; Hruby VJ and Balse PM, 2000; Golebiowski A et al., 2001). Various methodology for incorporating unnatural amino acids into proteins, using both *in vitro* and *in vivo* translation systems, to probe and/or improve protein structure and function are also disclosed in the literature (Dougherty DA, 2000). MCP-1 antagonists peptide mimics are known in the literature, without being highly homolohous to MCP-1 (Kaji M et al., 2001).

The present patent application discloses as MCP antagonists polypeptides or peptides comprising the amino acid sequence as defined above and an amino acid

sequence belonging to a protein sequence other than the corresponding MCP protein. This heterologous latter sequence should provide additional properties without impairing significatively the antagonistic activity, or proving GAG binding properties. Examples of such additional properties are an easier purification procedure, a longer lasting half-life in body fluids, or extracellular localization. This latter feature is of particular importance for defining a specific group of fusion or chimeric proteins included in the above definition since it allows the molecules defined as MCP antagonists in this patent application to be localized in the space where not only where the isolation and purification of these peptides is facilitated, but also where MCP proteins and their receptor naturally interact.

Additional protein sequences which can be used to generate the polypeptide or peptide of (c) are the ones of extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins. The choice of one or more of these sequences to be fused to the mutant of MCP protein is functional to specific use of said agent. As a general procedure, these fusion proteins can be produced by generating nucleic acid segments encoding them, using common genetic engineering techniques, and cloning in replicable vector of viral or plasmid origin which are used to modify a Prokaryotic or Eukaryotic host cell, using episomal or non-/homologously integrated vectors, as well as transformation-, infection-, or transfection-based technologies. These vectors should allow the expression of the fusion protein including the MCP antagonist in the prokaryotic or eukaryotic host cell under the control of their own transcriptional initiation/termination regulatory sequences, which are chosen to be constitutively active or inducible in said cell. A cell line substantially enriched in such cells can be then isolated to provide a stable cell line.

When the additional protein sequence, as in the case of the sequence of extracellular, export signal, or signal-peptide containing proteins, allows the MCP antagonist to be secreted in the extracellular space, the agent can be more easily collected and purified from cultured cells in view of further processing or, alternatively, the cells can be directly used or administered.

The polypeptides and the peptides of the present invention can be in other alternative forms which can be preferred according to the desired method of use and/or production, for example as active fractions, precursors, salts, derivatives, conjugates or complexes.

The term "active" means that such alternative compounds should maintain the functional features of the MCP mutants of the present invention, and should be as well pharmaceutically acceptable and useful.

The term "fraction" refers to any fragment of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the original polypeptide or peptide. Such molecules can result also from other modifications which do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical derivativization of peptides (acetylation or carboxylation), those made by modifying the pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes) of a peptide during its synthesis and processing or in further processing steps.

The "precursors" are compounds which can be converted into the compounds of present invention by metabolic and enzymatic processing prior or after the administration to the cells or to the body.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the peptides and polypeptides of the invention or their analogs.

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N-/ or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

Useful conjugates or complexes of the MCP antagonists of the present invention can be generated, using molecules and methods known in the art of the interaction with

receptor or other proteins (radioactive or fluorescent labels, biotin), therapeutic efficacy (cytotoxic agents), or improving the agents in terms of drug delivery efficacy, such as polyethylene glycol and other natural or synthetic polymers (Pillai O and Panchagnula R, 2001).

The compounds of the invention may be prepared by any well known procedure in the art, including recombinant DNA-related technologies described above, and chemical synthesis technologies.

Another object of the invention are the DNA molecules comprising the DNA sequences coding for the MCP mutants of the invention, including nucleotide sequences substantially the same. "Nucleotide sequences substantially the same" includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequences.

The invention also includes expression vectors which comprise the above DNAs, host cells transformed with such vectors and a process of preparation of MCP antagonists of the invention, through the culture in appropriate culture media of said transformed cells, and collecting the expressed protein.

The DNA sequence coding for the proteins of the invention can be inserted and ligated into a suitable plasmid. Once formed, the expression vector is introduced into a suitable host cell, which then expresses the vector(s) to yield the desired protein.

Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in eukaryotic cells (e.g. yeasts, insect or mammalian cells) or prokaryotic cells, using the appropriate expression vectors. Any method known in the art can be employed.

For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art. Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing the desired protein, an expression vector should also comprise specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding the desired protein in such a way as to permit gene expression and production of the protein. First in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA

polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

For Eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived form viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of the Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the protein of the invention is inserted into vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell.

The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to a auxotropic host, biocide resistance, e.g. antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the invention.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells, that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Once the vector(s) or DNA sequence containing the construct(s) has been prepared for expression the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells may be either prokaryotic or eukaryotic. Preferred are eukaryotic hosts, e.g. mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired proteins.

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid corresponding to the C-terminus of the peptide to be synthetized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the C-terminus to the N-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl2-Bzl (2,6-dichlorobenzyl) for the amino groups; NO2 (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino groups); and tBu (t-butyl) for the hydroxyl groups). After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method. Totally synthetic MCP proteins are disclosed in the literature (Brown et al., 1996).

Purification of the natural, synthetic or recombinant MCP antagonists of the invention can be carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies or affinity groups, which bind the target protein and which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by heparin or by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength. Alternatively, HPLC (High Performance Liquid Chromatography) can be used. The elution can be carried using a water-acetonitrile-based solvent commonly employed for protein purification. The invention includes purified preparations of the compounds of the invention. Purified preparations, as used herein, refers to the preparations which are at least 1%, preferably at least 5%, by dry weight of the compounds of the invention.

Another object of the present invention is the use of MCP proteins antagonists as above defined as medicaments, in particular as the active ingredients in pharmaceutical compositions (and formulated in combination with pharmaceutically acceptable carriers, excipients, stabilizers, or diluents) for treating or preventing diseases related to an undesirable activity of MCP proteins leading to an excessive migration and activation of leukocytes expressing their receptors, such as autoimmune and inflammatory diseases as well as bacterial and viral infections. Non-limitative examples of such diseases are the following: arthritis, rheumatoid arthritis (RA), psoriatic arthritis, osteoarthritis, systemic lupus erythematosus (SLE), systemic sclerosis, scleroderma, polymyositis, glomerulonephritis, fibrosis, lung fibrosis, allergic or hypersensitvity diseases, dermatitis, Type IV hypersensitivity also called delayed-type hypersensitivity or DTH, asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), Crohn's diseases, ulcerative colitis, multiple sclerosis, septic shock, HIV–infection, transplantation, graft-versus-host disease (GVHD) and atherosclerosis.

Another object of the present invention is, therefore, the method for treating or preventing any of the above mentioned diseases comprising the administration of an effective amount of an MCP antagonist of the invention.

The pharmaceutical compositions may contain, in addition to the MCP antagonist, suitable pharmaceutically acceptable carriers, biologically compatible vehicles and additives which are suitable for administration to an animal (for example, physiological saline) and eventually comprising auxiliaries (like excipients, stabilizers or diluents) which facilitate the processing of the active compounds into preparations which can be used pharmaceutically. The pharmaceutical compositions may be formulated in any acceptable way to meet the needs of the mode of administration. For example, the use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration, are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001).

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology. The effective amount will depend on the route of administration and the condition of the patient.

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

Any accepted mode of administration can be used and determined by those skilled in the art to establish the desired blood levels of the active ingredients. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Parenteral administration can be by bolus injection or by gradual perfusion over time. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran.

Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound together with the excipient. Compositions which can be administered rectally include suppositories.

It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art. The total dose required for each treatment may be administered by multiple doses or in a single dose. The pharmaceutical composition of the present invention may be administered alone or in conjunction with other therapeutics directed to the condition, or directed to other symptoms of the condition. Usually a daily dosage of active ingredient is comprised between 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage, which is the same, less than, or greater than the initial or previous dose administered to the individual.

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions , which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

## EXAMPLES

**Example 1: *in vitro* characterization of the non-heparin binding MCP-1 mutant MCP-1WT*2A**

Materials and methods

*Expression of the MCP-1 mutants MCP-1WT* and MCP-1WT*2A.*

MCP-1 mutants were generated by *in vitro* mutagenesis of the DNA sequence coding for human MCP-1 (hMCP-1; fig. 1; SEQ ID NO: 1), and in particular for the mature form of human MCP-1, corresponding to the segment 24-99 of the precursor molecule (SWISSPROT Acc. N° P13500).

According to literature (Paavola CD et al, 1998), MCP-1 is more easily purified from *E. coli* if an internal methionine of the MCP-1 coding sequence (amino acid 87 in the precursor and 64 in the mature protein) cloned in the expression vector, is replaced with an isoleucine. In this mutant, called MCP-1WT*, the substitution avoids the formation of undesirable MCP-1 species containing methionine-sulfoxide, without any significant effect on the binding and the activity of the protein.

At this scope, it was first generated a plasmid encoding for the "active" mutant MCP-1WT*, in which a methionine start codon is added at the N-terminal of sequence coding for human MCP-1 (24-99) mutated in the above indicated position. The resulting sequence, called MCP-1WT*, is expressed, by making use of a plasmid based on the pET3 plasmid (Paavola CD et al, 1998), as a protein containing 77 residues (fig. 1A; SEQ ID NO: 2).

The plasmid expressing MCP-1WT* was then further mutagenized by cloning a PCR fragment encoding for two Alanines instead of Arginine and Lysine in positions 41 and 42 of human MCP-1 precursor, in order to generate an MCP-1 mutant, MCP-1WT*2A, having the same length purification features of MCP-1WT* (fig. 1; SEQ ID NO:3).

All constructs were obtained and controlled by standard molecular biology technologies (PCR mutagenesis and amplification, DNA sequencing, restriction digestion) and maintained in the DH5alpha strain of *E. coli* during the cloning process. The coding sequences were chosen in order to have an optimal codon usage for expression in *E. coli* (Kane JF et al., 1995).

The pET3-based plasmids encoding for MCP-1WT* and MCP-1WT*2A were transferred in an *E. coli* BL21(pLys)-derived strain called TAP302 and the resulting strains were used to express the MCP-1 mutants as described (Paavola CD et al, 1998). This protocol includes the use of aminopeptidase to removes the N-terminal methionine, thus obtaining recombinant MCP-1 mutants having the same length of the natural mature form (76 amino acids; fig. 1B). The identity of the protein was verified by mass spectrometry.

*Chromatographic assays of MCP-1WT* and MCP-1WT*2A.*

MCP-1WT* and MCP-1WT*2A were loaded either onto a Heparin Sepharose column (using 100 micrograms of protein) or onto a SP Sepharose cation exchange column (using 15 milligrams of protein). In both cases the column was equilibrated in 10 mM $KPO_4$(pH 7.5) and the protein was eluted with a linear gradient of 0 - 1M NaCl in the same buffer.

*Heparin binding assay of MCP-1WT* and MCP-1WT*2A.*

Serial dilutions of MCP-1WT* mutants in Phosphate Buffer Saline (PBS) covering the range of 0.02-30 μM were incubated with 170 nM of [$^3$H]-heparin for 1 hour at 37 °C. Triplicates of 20 μl of each sample were transferred to a 96 well P81 Unifilter plate (Whatman Inc) fitted with a nitrocullose filter. The plate was washed three times with 200 μl of PBS using a vacuum pump to remove unbound labelled heparin. The scintillation fluid (50 μl) was added to each well and radioactivity counted (1 min/well) in a beta counter. Data were analysed using GraphPad Prism Software .

*Equilibrium competition receptor binding assays*

The assays were carried out on membranes from CHO transfectants expressing CCR2 using a Scintillation Proximity Assay (SPA) using [$^{125}$I]-MCP-1 as tracer. The radiolabelled chemokine (specific activity of 2200 mCi/mole) was generated from recombinant MCP-1 according to the [$^{125}$I] supplier (Amersham). Competitors were prepared by serial dilutions (range $10^{-6}$-$10^{-12}$ M) of the unlabelled MCP-1 mutant in the binding buffer (50 mM HEPES pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.15 M NaCl and 0.5% BSA).

Wheat germ SPA beads (Amersham) were solubilised in PBS to 50 mg/ml, and diluted in the binding buffer to a 10 mg/ml, and the final concentration in the assay was 0.25 mg/well. Membranes expressing CCR2 were stored at –80°C and diluted in the binding buffer to 80 μg/ml. Equal volumes of membrane and beads stocks were mixed before performing the assay to reduce background. The final membrane concentration was 2 μg/well and that of [$^{125}$I]- MCP-1 was 0.1nM. The plates were incubated at room temperature with agitation for 4 hours. Radioactivity was counted (1 min/well) in a beta counter. Data from triplicate samples were analysed using Grafit Software.

Results

Two mutants of MCP-1, whose sequence is based on the mature form of human MCP-1, were expressed in *E coli* (fig. 1). A first one, MCP-1WT*, corresponds to mature human MCP-1 precursor with a mutation known to allow an easier purification without interfering with the binding properties and activity typical of MCP-1 (Paavola CD et al, 1998). On the basis of the sequence of this "active" mutant, a second mutant, MCP-1WT*2A was expressed in which a dibasic site at the N-terminal was additionally replaced with Alanine residues.

The effect of this latter substitution on the MCP-1 properties was first tested by chromatography, comparing the elution profiles of the two mutants. Using heparin chromatography, the concentration of NaCl required to elute MCP-1WT* was 0.54 M NaCl whilst MCP-1WT*2A was eluted at 0.24 M NaCl. A smaller difference was measured using cation exchange chromatography on a SP Sepharose column (0.55 M NaCl against 0.27 M NaCl). The difference in NaCl concentration obtained on cation exchange chromatography is subtracted from that obtained on heparin chromatography, and since this is value is positive (0.02 M), a specific interaction with heparin is identified as being associated to the dibasic site mutated in MCP-1WT*2A.

A direct measure of binding to heparin was then performed using tritiated heparin and serial dilution of the *E. coli* expressed MCP-1 mutants (fig. 2). The resulting complexes were isolated by exposing the reactions to nitrocellulose filters, which are capable to retain protein efficiently, therefore allowing a direct evaluation of amount of the radiolabelled heparin bound to the protein. This approach confirmed that the heparin binding properties of MCP-1WT*2A were significantly reduced compared to MCP-1WT*.

Finally, an equilibrium competition receptor binding assay was performed to demonstrate the effect of the reduced heparin binding properties of MCP-1WT*2A on the binding of the specific receptor CCR2 (fig. 3). Samples containing radiolabelled MCP-1 mixed with serial dilutions of one of the two mutants, or of MCP-1, were incubated with membranes prepared from CHO cells stably expressing CCR2. Whilst MCP-1WT* and MCP-1 protein showed an almost identical binding profile, the MCP-1WT*2A mutant shows a 20 fold reduction in affinity for CCR2, since it has an $IC_{50}$ of 1.73 $\pm$ 0.6 nM, compared to 0.08 $\pm$ 0.045 nM for the other two tested proteins. However, high affinity is retained also in this heparin-binding defective MCP-1 mutant.

**Example 2: *in vivo* characterization of a non-heparin binding MCP-1 mutant.**

Materials and methods

*Chemotaxis assay*

The assay was carried out using human macrophage (THP-1 cells) and 24-well transwell chemotaxis chambers (Costar) fitted with 5 μm pore size membranes (Neuroprobe). The recombinant MCP-1 proteins were serially diluted (range of $10^{-6}$-$10^{-12}$ M) in 600 μl of RPMI medium containing 5% inactivated fetal calf serum (FCS), 2 mM glutamine and 25 mM HEPES(pH 7.2). These samples were placed in the lower wells, whilst THP-1 cells (100 μl of a cell suspension at 10 x $10^6$ cells/ml in the same medium) were placed in the inserts. The chamber was incubated for 3 hours at 37 °C under 5% $CO_2$. The samples were then removed, transferred to a 1.5 ml tube, and centrifuged at 200x g for 5 minutes. The pelleted cells were resuspended in 100 ml PBS and counted in a Coulter counter (Beckman). The data were analyzed using GraphPad Prism software.

*Peritoneal cellular recruitment*

Cellular recruitment was induced by intraperitoneal injection of female BALB/c mice of 8 to 12 wk of age of 10 μg of the recombinant MCP-1 protein diluted in 0.2-ml sterile, Lipopolysaccharide-free saline. When the antagonistic properties of MCP-1WT*2A were tested, the indicated amounts of the protein, diluted in 0.2 ml of the same sterile solution, were administered 30 minutes prior to the agonist administration. Mice were sacrificed by aerosolized $CO_2$ 16 hours after the administration of the agonist, and peritoneal lavage was performed with 5 ml PBS three times. The lavages were pooled and centrifuged at 600x g for 10 minutes, and the pelleted cells were resuspended in a final volume of 1 ml and total elicited leukocytes were counted with an hemacytometer.

*Delayed Contact Hypersensitivity Assay*

The mouse ear-swelling test to measure contact hypersensitivity as performed as has described (Garrigue JL et al., 1994). Briefly, mice were pre-sensitized topically by applying 25 μl of 0.5% 2,4-dinitrofluorobenzene (DNFB; Sigma Chemical Co.) solution in ace-tone/ olive oil (4:1) to the shaved abdomen. Five days later, 20 μl of 0.2% DNFB

in the same vehicle was applied to the right ears, and vehicle alone to the left ears. Mice were treated daily from Day 5 to 9 with an intraperitoneal administration of either 0.5 mg/kg MCP-1WT* or vehicle only in the control group. The first treatment was administered 1 hour prior to the DNFB challenge. Ear thickness was measured with a dial thickness gauge (Mitutoyo Corp.), and ear swelling was estimated by subtracting the pre-challenge from the post-challenge value, and by further subtracting any swelling detected in the vehicle-challenged contralateral ear.

*Bleomycin induced lung fibrosis*

C57BL/6 female mice received bleomycin (3.75 U/kg in 25 µl saline) intra-tracheally (day 0). One our after the instillation of bleomycin, test animals received either 0.25 mg/kg MCP-1WT*2A or saline intra-peritoneally. This treatment was given daily and continued for 10 days. The body weight loss and percentage of mortality were recorded daily. At day 10, all mice were sacrified by $CO_2$ asphyxiation. Four lung lobes were placed at –80°C for measurement of hydroxyproline levels as an indication of collagen deposition as well as one lobe processed for histological determination of pulmonary fibrosis. Total lung collagen was determined by the analysis of hydroxyproline. Briefly, lungs were homogenized in Tris-HCl, pH 7.6, with a Tissue Tearor followed by incubation in Amberlite overnight at 115°C. Citrate/acetate buffer, isopropanol, chloramine-T and DAB solutions were added to the samples and left for 30 min at 60° C. Samples were cooled for 10 min and read at 560 nm on spectrophotometer. Pulmonary fibrosis was determined histologically by fixation of the right lung lobe in 10% formalin, followed by embedding in paraffin, sectioning, and staining with Masson's trichrome solution. Histological changes were examined by light microscopy. Morphological evaluation of bleomycin-induced lung inflammation and fibrosis was performed using a semi-quantitative scoring method.

Results

The properties of MCP-1WT*2A were then tested by making use of cell- and animal-based assay to verify if any agonistic or antagonistic activity on MCP-1 can be associated to this heparin-binding defective mutant.

The results obtained in a chemotaxis assay on human macrophages (THP-1 cells) correspond well with those obtained in the receptor binding assay described above. MCP-1WT*2A was able to induce a robust response (6-fold over baseline) of

THP-1 chemotaxis although maximum activity was observed at 10 nM compared to 1 nM for the wild type proteins, which induced a 9-fold increase over baseline (Fig. 4).

The activity of MCP-1WT*2A as MCP-1 antagonist or agonist were evaluated using a peritoneal cellular recruitment assay (fig. 5). When MCP-1WT* and MCP-1WT* are administered, the latter mutant was unable to induce cellular recruitment into the peritoneum at the dose (10 μg/mouse) that recombinant MCP-1WT*, like the natural protein, causes substantial recruitment. Furthermore, if MCP-1WT*2A is administered 30 minutes prior to the administration of MCP-1WT*, the cellular recruitment induced by MCP-1WT* is significantly antagonized in a dose dependent manner. Therefore, the abrogation of GAG-binding in MCP-1 produces an antagonist of MCP-1 capable of inhibiting *in vivo* the cellular recruitment induced by MCP-1.

Further evidences of the *in vivo* properties of MCP-1WT*2A were obtained by making use of two other animal models.

A first assay was intended to assess the properties of this molecule in a skin inflammation model. Delayed contact hypersensitivity is a hapten-specific skin inflammation mediated by T cells which generates a measurable swelling. The ear skin of mice was challenged making use of the contact sensitizer 2,4-dinitrofluorobenzene (DNFB) as hapten. The consequent swelling was significantly lower in mice treated with an intraperitoneal administration of MCP-1WT*2A (starting at the time of challenge with DNFB), when compared to the effect observed in mice treated with vehicle alone, throughout the treatment period (Fig. 6).

A second model was intended to assess the properties of MCP-1WT*2A in a lung inflammation and fibrosis model. Intra-tracheal instillation of Bleomycin in mice results in lung inflammation and fibrosis within 7 to 10 days respectively, with a marked accumulation of collagen in the lungs as well as a rapid decrease of weight. This latter easily measurable index was recorded throughout the 10 days after the exposure to bleomycin in mice treated 1 hour later with an intraperitoneal administration of MCP-1WT*2A or only the vehicle, and further compared to a control group non treated with Bleomycin (Fig. 7). As it is clearly evident starting from day 2, vehicle control treated mice lose a significantly higher amount of weight than MCP-1WT*2A treated mice.

Lung fibrosis and inflammation was evaluated after sacrificing the animals at day 10 (Fig. 8) using two different methods. It is known that bleomycin increases lung hydroxyproline synthesis proportionally to collagen synthesis and fibrosis (Madtes DK et al., 1999). The hydroxyproline levels measured in mice treated with vehicle only

were significatively higher than the levels measured in the group receiving an intraperitoneal administration of MCP-1WT*2A. Indeed, the levels in this latter group were comparable to non bleomycin treated mice. Another semi-quantitative histological assessment confirmed the significant reduction in total levels of fibrosis in the MCP-1WT*2A treated group versus control.

Given that the dibasic site mutated in the examples of the present invention, together with the other residues known to be involved in MCP-1 binding to GAGs such as Histidine 66 and Lysine 58 (Chakravarty L et al., 1999), is conserved in all MCPs (Blaszczyk J et al., 2000; Proost P et al., 1996), other MCPs-based mutants having antagonistic activities can be designed on the basis of the findings of this patent application. In particular, these MCP-1/ -2/ -3 / -4 antagonists can be double mutants of human mature MCP-1 (SEQ ID NO: 4), MCP-2 (SEQ ID NO: 5), MCP-3 (SEQ ID NO: 6), and MCP-4 (SEQ ID NO: 7) in the positions 18 and 19, 18 and 58 (or 66), 19 and 58 (or 66), as well as triple mutants in the positions 18, 19 and 58 (or 66; the numbering corresponds the one given for human mature MCP-1). Other basic residues which can be mutated additionally to the ones in positions 18 and 19 are the basic residues identified as highly conserved in all human MCP proteins (residues 24, 44, 49, 75; fig. 1B)

**TABLE I**

| Amino Acid | Synonymous Group | More Preferred Synonymous Groups |
|---|---|---|
| Ser | Gly, Ala, Ser, Thr, Pro | Thr, Ser |
| Arg | Asn, Lys, Gln, Arg, His | Arg, Lys, His |
| Leu | Phe, Ile, Val, Leu, Met | Ile, Val, Leu, Met |
| Pro | Gly, Ala, Ser, Thr, Pro | Pro |
| Thr | Gly, Ala, Ser, Thr, Pro | Thr, Ser |
| Ala | Gly, Thr, Pro, Ala, Ser | Gly, Ala |
| Val | Met, Phe, Ile, Leu, Val | Met, Ile, Val, Leu |
| Gly | Ala, Thr, Pro, Ser, Gly | Gly, Ala |
| Ile | Phe, Ile, Val, Leu, Met | Ile, Val, Leu, Met |
| Phe | Trp, Phe,Tyr | Tyr, Phe |
| Tyr | Trp, Phe,Tyr | Phe, Tyr |
| Cys | Ser, Thr, Cys | Cys |
| His | Asn, Lys, Gln, Arg, His | Arg, Lys, His |
| Gln | Glu, Asn, Asp, Gln | Asn, Gln |
| Asn | Glu, Asn, Asp, Gln | Asn, Gln |
| Lys | Asn, Lys, Gln, Arg, His | Arg, Lys, His |
| Asp | Glu, Asn, Asp, Gln | Asp, Glu |
| Glu | Glu, Asn, Asp, Gln | Asp, Glu |
| Met | Phe, Ile, Val, Leu, Met | Ile, Val, Leu, Met |
| Trp | Trp, Phe,Tyr | Trp |

**TABLE II**

| Amino Acid | Synonymous Group |
|---|---|
| Ser | D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys |
| Arg | D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-.Met, D-Ile, Om, D-Om |
| Leu | D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met |
| Pro | D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid |
| Thr | D-Thr, Ser, D-Ser, allo-Thr, Met,D-Met, Met(O), D-Met(O), Val, D-Val |
| Ala | D-Ala, Gly, Aib, B-Ala, Acp, L-Cys, D-Cys |
| Val | D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG |
| Gly | Ala, D-Ala, Pro, D-Pro, Aib, .beta.-Ala, Acp |
| Ile | D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met |
| Phe | D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa |
| Tyr | D-Tyr, Phe, D-Phe, L-Dopa, His, D-His |
| Cys | D-Cys, S--Me--Cys, Met, D-Met, Thr, D-Thr |
| Gln | D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp |
| Asn | D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln |
| Lys | D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Om, D-Om |
| Asp | D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln |
| Glu | D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln |
| Met | D-Met, S--Me--Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val |

## REFERENCES


Ali S et al., Biochem J , 358: 737-745, 2001.

Baggiolini M et al., Annu Rev Immunol, 15: 675-705, 1997.

Baggiolini M, J Intern Med, 250: 91-104, 2001.

Blaszczyk J et al., Biochemistry, 39:14075-14081, 2000.

Chakravarty L et al., J Biol Chem, 273: 29641-29647, 1998.

Cleland JL et al., Curr Opin Biotechnol,12: 212-9, 2001.

Douglas MS et al., Immunology, 92: 512-518, 1997.

Dougherty DA, Curr Opin Chem Bio, 4: 645-52, 2000.

Fernandez EJ and Lolis E, Annu Rev Pharmacol Toxicol, 42:469-499, 2002.

Godessart N and Kunkel SL, Curr Opin Immunol, 13: 670-675, 2001.

Golebiowski A et al., Curr Opin Drug Discov Devel, 4: 428-34, 2001.

Gong J and Clark-Lewis I, J Exp Med 181: 631-640, 1995.

Gosling J et al., J Clin Invest, 103: 773-778, 1999.

Gu L et al., Chem Immunol, 72: 7-29, 1999.

Gu L et al., Nature, 404: 407-411, 2000.

Handel TM et al., Biochemistry,35 : 6569-6584, 1996.

Hemmerich S et al., Biochemistry, 38: 13013-13025, 1999.

Hoogewerf AJ et al., Biochemistry, 36: 13570-13578, 1997.

Hruby VJ and Balse PM, Curr Med Chem, 7:945-70, 2000

Kuschert G et al., Biochemistry 38: 12959-12968, 1999.

Loetscher P and Clark-Lewis I, J Leukoc Biol, 69: 881-884, 2001.

Lortat-Jacob H et al,, Proc Natl Acad Sci U S A, 99: 1229-1234, 2002.

Lu B et al., J Exp Med, 187: 601-608, 1998.

Lubkowski J et al., Nat Struct Biol, 4 : 64-69, 1997.

Luo B and Prestwich GD, Exp Opin Ther Patents, 11: 1395-1410, 2001.

Madtes DK et al., Am J Respir Cell Mol Biol, 20: 924-934, 1999.

Murphy LR et al., Protein Eng, 13:149-52, 2000.

Paavola CD et al., J Biol Chem, 273: 33157-33165, 1998.

Patel D et al., Clin Immunol, 99: 43-52, 2001.

Pillai O and Panchagnula R, Cur Opin Chem Biol, 5: 447-451, 2001

Proost P et al., J Leukoc Biol, 59 (1):67-74, 1996.

Rogov SI and Nekrasov AN, Protein Eng, 14: 459-463, 2001.

Sawyer TK, in "Structure Based Drug Design", edited by Veerapandian P, Marcel Dekker Inc., pg. 557-663, 1997.

Seet BT et al., Proc Natl Acad Sci U S A, 98: 9008-9013, 2001.

Steitz SA et al., FEBS Lett , 430: 158-164, 1998.

Villain M et al., Chem Biol, 8(7):673-9, 2001.

Zhang Y et al,, Methods 10: 93-103, 1996.

## CLAIMS

1. Antagonists of MCP proteins consisting of mutants of MCP proteins in which the following combinations of basic residues, numbered on the sequence of human mature MCP-1, are substituted to Alanine, Glycine, Serine, Threonine, Proline, Aspartic acid, or Asparagine:
   a) 18 and 19;
   b) 18 and/or 19, together with 58;
   c) 18 and/or 19, together with 66;
   d) 18 and/or 19, together with 58 and 66;
   e) 18 and/or 19, together with one or more of the following: 24, 44, 49, 75.
2. The antagonist of claim 1 wherein the positions 18 and 19 are substituted with Alanine.
3. The antagonist of claim 1 or 2 wherein the MCP proteins are human MCP-1, human MCP-2, human MCP-3, or human MCP-4.
4. The antagonist of claim 1 or 2 wherein the MCP proteins are proteins having at least 70% of homology with human mature MCP-1, MCP-2, MCP-3, or MCP-4.
5. The antagonist of claim 4 having the sequence corresponding to SEQ ID NO: 3.
6. Antagonist of MCP proteins selected from:
   a) active mutants of the antagonists of MCP proteins of claims from 1 to 5, in which one or more amino acid residues have been added, deleted, or substituted without interfering with the antagonistic activity;
   b) peptide mimetics designed on the sequence and/or the structure of polypeptides or peptides of (a);
   c) polypeptides or peptides comprising the amino acid sequence of (a) or (b), and an amino acid sequence belonging to a protein sequence other than the corresponding MCP protein;
   d) active fractions, precursors, salts, or derivatives of (a), (b), or (c).
7. The MCP antagonists of claim 6, wherein the polypeptide or peptide of (c) comprises the amino acid sequence belonging to one or more of these protein sequences: extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins.

8. The MCP antagonists of claim 6 or 7, wherein said antagonist is in the form of active conjugate or complex with a molecule chosen amongst radioactive labels, biotin, fluorescent labels, cytotoxic agents, drug delivery agents.
9. DNA molecules comprising the DNA sequences coding for the MCP antagonists of claims from 1 to 7, including nucleotide sequences substantially the same.
10. Expression vectors comprising the DNA molecules of claim 9.
11. Host cells transformed with vectors of claim 10.
12. Process of preparation of MCP antagonists of claims from 1 to 8, comprising culturing the transformed cells of claim 11 and collecting the expressed proteins.
13. Use of the MCP antagonists of claims from 1 to 8 as active ingredients in pharmaceutical compositions for the treatment or prevention of diseases related to excessive leukocyte migration and activation.
14. The use of claim 13 wherein the disease is an inflammatory disease, an autoimmune disease or an infection.
15. Pharmaceutical composition containing a MCP antagonist of claims from 1 to 8 as active ingredient.
16. Method for the treatment or prevention of diseases related to excessive leukocyte migration and activation, comprising the administration of an effective amount of an MCP antagonist of claims from 1 to 8.
17. The method of claim 16 wherein the disease is an inflammatory disease, an autoimmune disease or an infection.

## ABSTRACT

Novel antagonists of MCP proteins, in particular of MCP-1 protein, can be obtained by generating MCP mutants in which at least a GAG binding site located at the N-terminal of MCP proteins is eliminated following non-conservative substitutions. Compounds prepared in accordance with the present invention can be used to inhibit the migration and activation of leukocytes expressing their receptors, thereby providing useful therapeutic compositions for use in the treatment or prevention of diseases related to excessive leukocyte migration and activation, such as inflammation and autoimmune diseases.

## Figure 1

### A)

```
hMCP-1       1 MKVSAALLCL LLIAATFIPQ GLAQPDAINA PVTCCYNFTN RKISVQRLAS 50

MCP-1WT*     1                          MQPDAINA PVTCCYNFTN RKISVQRLAS 28
MCP-1WT*2A   1                          MQPDAINA PVTCCYNFTN AAISVQRLAS 28

hMCP1       51 YRRITSSKCP KEAVIFKTIV AKEICADPKQ KWVQDSMDHL DKQTQTPKT 99

MCP-1WT*    29 YRRITSSKCP KEAVIFKTIV AKEICADPKQ KWVQDSIDHL DKQTQTPKT 77
MCP-1WT*2A  29 YRRITSSKCP KEAVIFKTIV AKEICADPKQ KWVQDSIDHL DKQTQTPKT 77
```

### B)

```
hMCP-1    1 QPDAINAPVT CCYNFTNRKI SVQRLASYRR ITSSKCPKEA VIFKTIVAKE 50
hMCP-2    1 QPDSVSIPIT CCFNVINRKI PIQRLESYTR ITNIQCPKEA VIFKTKRGKE 50
hMCP-3    1 QPVGINTSTT CCYRFINKKI PKQRLESYRR TTSSHCPREA VIFKTKLDKE 50
hMCP-4    1 QPDALNVPST CCFTFSSKKI SLQRLKSY-V ITTSRCPQKA VIFRTKLGKE 49

hMCP-1   51 ICADPKQKWV QDSMDHLDKQ TQTPKT 76
hMCP-2   51 VCADPKERWV RDSMKHLDQI FQNLKP 76
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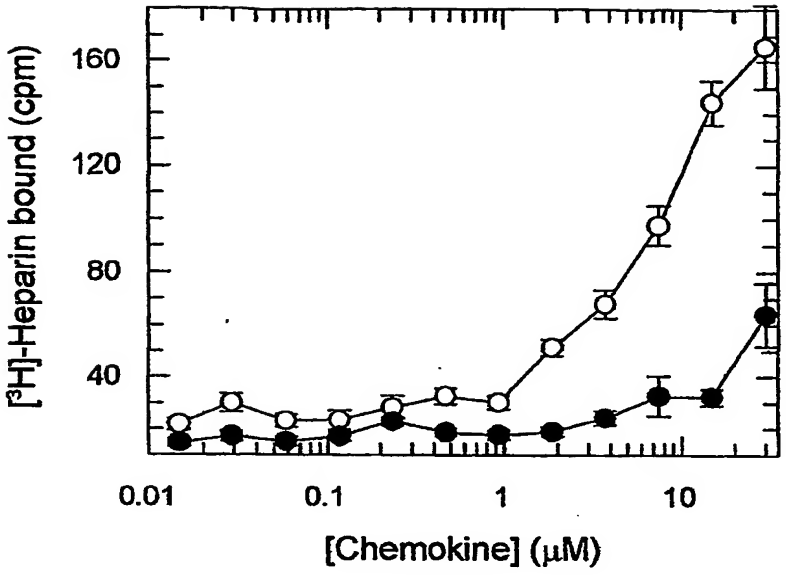
**Figure 2**



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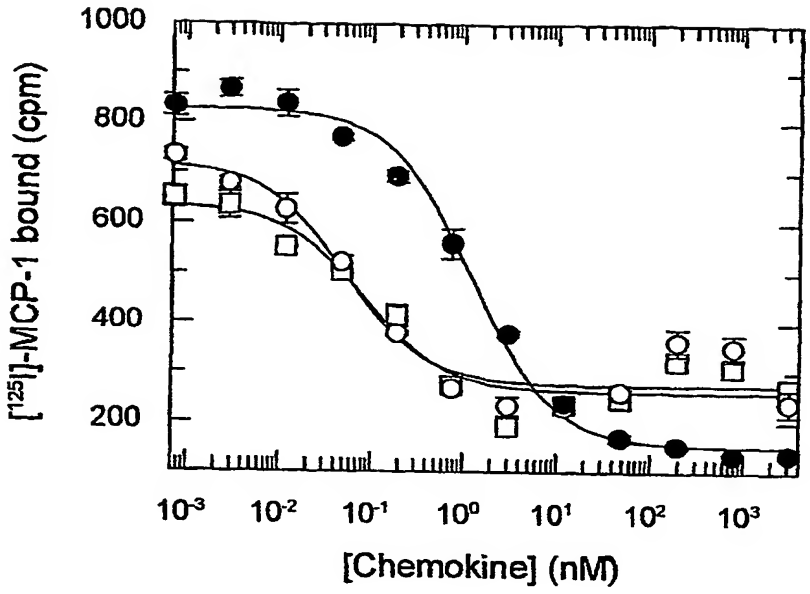
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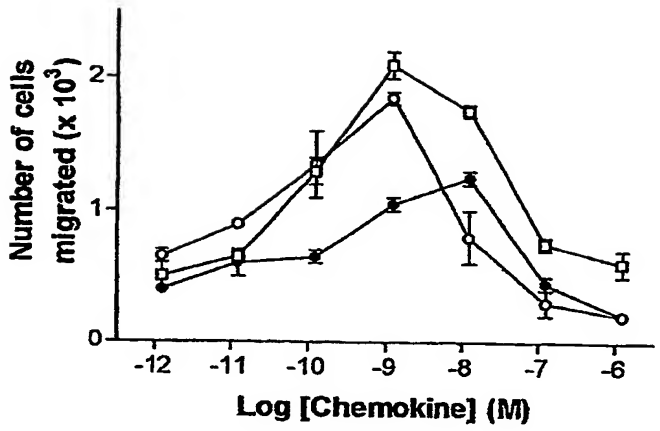
101
102
103
[Chemokine] (nM)

**Figure 4**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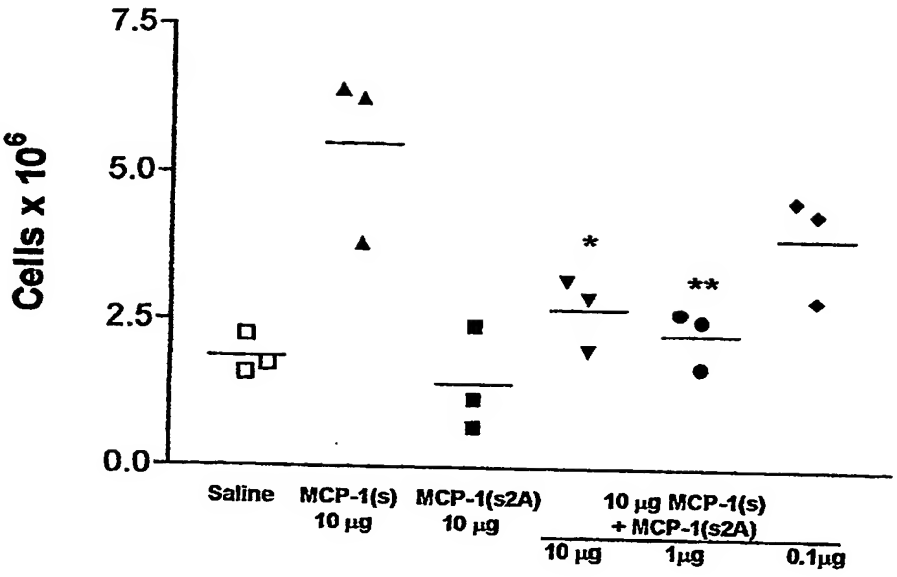
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Cells x 10^6
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10 µg MCP-1(s) + MCP-1(s2A)
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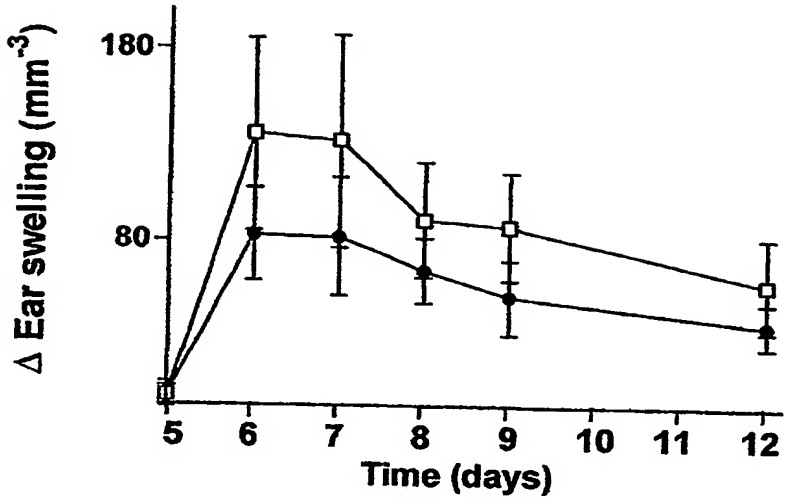
1µg
0.1µg

**Figure 6**



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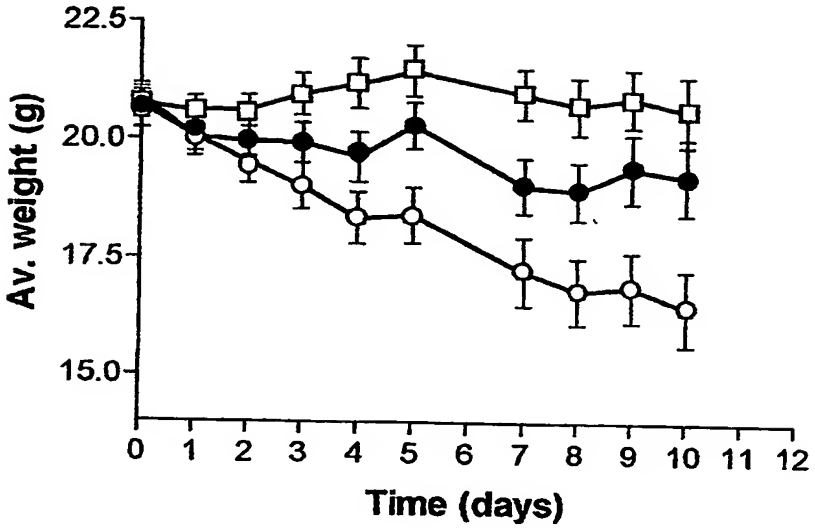

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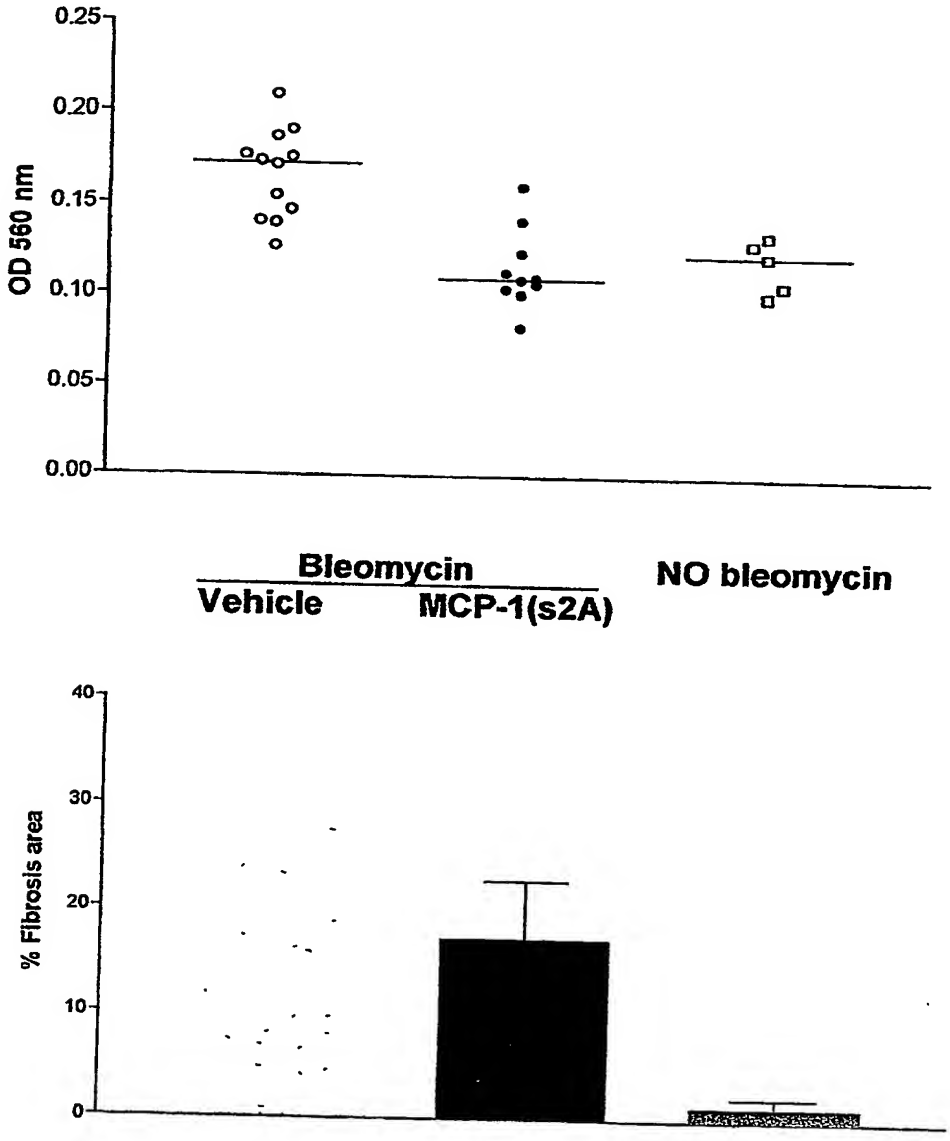
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